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Neuron-Specific Phosphoproteins as Biochemical Indicators of Neurotoxicity Effects of Acute Administration of Trimethyltin to the Adult Rat

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16. ABSTRACT

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Neuron-Specific Phosphoproteins as Biochemical Indicators of Neurotoxicity: Effects of Acute Administration of Trimethyltin to the Adult Rat[1]

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ABSTRACT

The cytoarchitecture of the adult central nervous system is expressed by proteins specific to individual cell types. In this investigation, a subclass of these proteins, the neuron-specific phosphoproteins, was examined after the administration of trimethyltin (TMT), a neurotoxicant which preferentially damages neurons in limbic structures. After acute administration of TMT (0.0–9.0 mg/kg i.v.), effects on neuronal phosphoproteins were examined by three separate techniques: 1) endogenous phosphorylation of total synaptic membrane proteins; 2) radiometric assay of synapsin I, a neuron-specific phosphoprotein associated with synaptic vesicles; and 3) radioimmunoassay of synapsin I and protein III, another synapse specific, synaptic vesicle-localized phosphoprotein. All three procedures gave similar results. TMT caused dose- and time-dependent decreases in hippocampal phosphoproteins. These effects were large in magnitude and were still evident 14 weeks after exposure to TMT. Microdissection of slices of dorsal hippocampus did not reveal significant regional differences in the extent to which TMT affected synapsin I. Phosphoproteins in frontal cortex, unlike those in hippocampus, were not affected by TMT. Our findings are consistent with the neuropathological effects of this compound and suggest that neuron-specific phosphoproteins may be useful biochemical indicators of neurotoxicity.

The cellular heterogeneity of the developing and mature nervous system is expressed by proteins specific to individual cell types (for reviews see: Zomzely-Neurath and Keller, 1977; Bock, 1978; Raff *et al.*, 1979; Kennedy, 1982; Schachner, 1982; Nestler and Greengard, 1983). The availability of techniques for identifying and, in some cases, measuring these proteins makes it feasible to characterize nervous tissue cell types on the basis of biochemical and immunochemical as well as morphological criteria. Our interest in nervous system-specific proteins is based on extensive neuropathological data which indicate that neurotoxic effects of environmental pollutants are often highly selective (Spencer and Schaumberg, 1980). For example, toxicants are known preferentially to affect specific cell types (*e.g.*, neurons and oligodendroglia) or subsets of a given cell type (*e.g.*, specific types of neurons), often within only a discrete region of the nervous system. The possibility that the response of an organism to neurotoxic insult could be characterized and quantified by changes in nervous system-specific proteins led us to propose the use of these proteins as broadly applicable biochemical indicators of neurotoxicity (O'Callaghan and Miller, 1983). In order to evaluate this hypothesis, we are assessing the effects of known neurotoxicants on proteins specific to cellular and subcellular elements of the mature and developing nervous system.

The organometallic compound, TMT, is neurotoxic to the adult rat. Acute or chronic exposure to TMT, while sparing myelin (Brown *et al.*, 1979), damages the neuronal component of the CNS (Brown *et al.*, 1979; Bouldin *et al.*, 1981; Chang and Dyer, 1983). Evidence from both light microscopic (Brown *et al.*, 1979; Chang and Dyer, 1983) and ultrastructural (Bouldin *et al.*, 1981) studies indicate that the cytopathologic effects of TMT are most pronounced in limbic system neurons and are accompanied by neurobehavioral alterations indicative of limbic system dysfunction (Walsh *et al.*, 1982). This regional and cell-type profile of TMT-induced neurotoxicity make this compound a useful tool for validating the use of nervous system-specific proteins as biochemical indicators of neurotoxicity.

In the present study we examined the effects of TMT on a subclass of nervous system-specific proteins, the neuronal phosphoproteins (for reviews see Nestler and Greengard, 1983,

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[1] This paper has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use. A preliminary report of this investigation appeared in Soc. Neurosci. Abstr. 9: 267, 1983.

ABBREVIATIONS: TMT, trimethyltin; CNS, central nervous system; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; cAMP, cyclic AMP.

1984), including the nerve terminal specific phosphoproteins, synapsin I (De Camilli *et al.*, 1983a,b; Huttner *et al.*, 1983) and protein III (Huang *et al.*, 1982; Tsou and Greengard, 1982; Browning *et al.*, 1982). Of the neuron-specific phosphoproteins, synapsin I is the most well characterized (for reviews see Greengard, 1981; Nestler and Greengard, 1983, 1984) and it was, therefore, the focus of the present investigation. Measurements were limited to two regions of the CNS, hippocampus and frontal cortex. By examining neuronal phosphoproteins in an extralimbic region (frontal cortex) as well as a limbic structure known to be severely damaged by TMT (hippocampus), we expected to find changes consistent with the effects of this compound on the morphology of these brain regions. On the basis of evidence obtained from both biochemical and immunochemical assays, we report that acute administration of TMT results in dose- and time-related decreases in hippocampal phosphoproteins without causing similar changes in phosphoproteins from frontal cortex. In the following paper, we demonstrate that the neurotoxic effects of TMT in the developing rat are characterized by alterations in nervous system-specific proteins, neurobehavioral function and morphology (Miller and O'Callaghan, 1984).

Materials and Methods

Materials. [γ-^{32}P]ATP (10–40 Ci/mmol was purchased from New England Nuclear (Boston, MA). Piperazine-N,N′-bis(2-ethanesulfonic acid) and Tris were obtained from Calbiochem (San Diego, CA). Reagents used for SDS-PAGE were of electrophoretic grade and were purchased from Bio-Rad Laboratories (Richmond, CA). Sucrose (grade II) and catalytic subunit (beef heart) were from Sigma Chemical Co. (St. Louis, MO). Synapsin I, protein III and corresponding antisera (rabbit) were the generous gifts of Drs. M. Browning, E. Perdahl and Jesse Chan, Rockefeller University. The specificity of the antisera against rat antigen has been demonstrated by 1) the double agar immunodiffusion test, 2) immunoprecipitation from brain extracts and 3) radioimmunolabeling of SDS-polyacrylamide gels (see De Camilli *et al.*, 1979; Goelz *et al.*, 1981 for additional details). Other materials used in the radioimmunoassays were from the sources described (Goelz *et al.*, 1981). TMT hydroxide was obtained from ICN Pharmaceuticals, Inc. (Plainview, NY).

Subjects. Male, Long-Evans rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighing 175 to 225 g were housed individually in a temperature (22°C ± 2)- and humidity (50% ± 10)-controlled colony room maintained on a 12-hr light/12-hr dark cycle beginning at 6:00 A.M.

Dosing. Rats were injected i.v. *via* tail vein with saline (0.9%) or TMT hydroxide (3.0, 6.0 or 9.0 mg/kg) dissolved in saline. Doses of TMT are expressed as the free base and were administered in a volume of 1.0 ml/kg b.wt. Except where brains were prepared for histology (see below), all subjects were killed by decapitation.

Brain dissection. Immediately after decapitation whole brains were excised from the skull and weighed. The hippocampus and frontal cortex were then obtained by free-hand dissection and weighed. Care was taken to remove overlying white matter from hippocampal samples. The frontal cortex was defined as the region between the anterior pole of the brain and the anterior commissure. Brain regions obtained from control subjects were always prepared in tandem with those obtained from subjects receiving TMT. In one experiment, 700-μ thick transverse sections of the dorsal hippocampus (unilateral) were prepared with a McIlwain Tissue Chopper (Brinkman Instruments, Westbury, NY). Each slice was subdivided into areas CA1, CA3 and fascia dentata as described by Danscher *et al.* (1976) with the exception that the regions designated II and III were combined. When dissected in this manner areas CA1 and CA3 would each contain a portion of CA2; areas CA3 and CA4 as well as the molecular and granular layers of the dentate gyrus would comprise the area designated fascia dentata (for a discussion of nomenclature, see Shepherd, 1979). All dissections were performed on a refrigerated plate maintained at 0–4°C (Thermoelectrics Unlimited, Wilmington, DL). Areas obtained from five slices were pooled before acid extraction of membrane proteins (see below).

Histology. Three weeks after the administration of saline or TMT (9.0 mg/kg), rats were anesthetized (3.5 ml/kg of Chloropent; Fort Dodge Laboratories, Fort Dodge, IA) and then were killed by transcardial perfusion with isotonic saline, followed by 10% neutral-buffered Formalin. Brains were then removed and sliced in saggital section at midline. Beginning at midline, sequential saggital sections 10-μ thick were cut at 100-μ intervals from the left side of each brain. Beginning at the anterior pole of the brain, sequential coronal sections 10-μ thick were cut at 50-μ intervals from the right side of each brain for a total anterior-posterior distance of approximately 5 mm. Sections were stained with cresyl violet.

Endogenous phosphorylation of synaptic proteins. Individual (bilateral) samples of hippocampus and frontal cortex were homogenized in 50 volumes of ice-cold 0.32 M sucrose, 5 mM Tris HCl (pH 7.2), 5 μM $MgSO_4$, using a glass-Teflon homogenizer (0.18–0.22 mm rated clearance, Arthur H. Thomas, Philadelphia, PA) and a crude synaptic membrane fraction (M_1) was prepared as described (De Robertis *et al.*, 1967). These samples were then preincubated at 0°C for 1 hr for the purpose of converting endogenous phosphoproteins entirely to the dephosphorylated form by the action of endogenous phosphoprotein phosphatases (Forn and Greengard, 1978; Sieghart *et al.*, 1979; Lohmann *et al.*, 1978). Endogenous phosphorylation of synaptic membrane proteins prepared from hippocampus and frontal cortex was assayed as described previously (O'Callaghan *et al.*, 1983). The standard assay contained: 100 μg of membrane proteins, 10 mM piperazine-N,N′-bis(2-ethane-sulfonic acid) (pH 6.8), 10 mM $MgCl_2$ and 5 μM ATP containing [γ-^{32}P] ATP (1×10^7 dpm/nmol) in the absence or presence of 10 μM cAMP (to detect endogenous synapsin I present in the M_1 fraction). The final volume was 200 μl and assays were conducted at 30°C. The phosphorylation reaction was initiated by addition of a 25-μl aliquot of the M_1 fraction and was terminated 15 sec later by the addition of 100 μl of an SDS-PAGE sample buffer (O'Callaghan *et al.*, 1983). All samples were heated to 90–95°C before SDS-PAGE.

Acid extraction and phosphorylation of synapsin I. Synapsin I was extracted from total membrane proteins using a modification of the procedure described by Goelz *et al.* (1981). Using microfuge tubes in combination with a custom-fitted Teflon pestle, samples of hippocampus (unilateral and dissected contralateral) and frontal cortex (unilateral) were homogenized in 10 volumes of ice-cold 0.32 M sucrose, 5 mM Tris HCl (pH 7.2) and 5 μM $MgSO_4$. The homogenates were allowed to stand on ice for 60 min for the purpose of converting endogenous synapsin I entirely to the dephosphorylated form (see above). Ice-cold piperazine-N,N′-bis(2-ethanesulfonic acid), pH 7.4, containing 5 mM Zn $(OAc)_2$ was then added to each homogenate at a volume equal to 10 volumes of the original sample. Following this addition, the samples were centrifuged at $3000 \times g$ for 10 min and the supernatants were discarded. In the original studies by Greengard and co-workers (Forn and Greengard, 1978; Sieghart *et al.*, 1979) Zn $(OAc)_2$ was included in the initial homogenizing buffer to inhibit phosphoprotein phosphatases and protein kinases. In the present study, however, we employed Zn $(OAc)_2$ to aggregate membranes (Forn and Greengard, 1978) in order to effect a more uniform recovery of the pelleted samples. After centrifugation, each pellet was rehomogenized in 11 mM ice-cold citric acid (600 μl for unilateral samples of hippocampus and frontal cortex and 200 μl for each region dissected from slices of hippocampus) and centrifuged at $8000 \times g$ for 5 min. Aliquots of the synapsin I-containing supernatants were removed and neutralized with 0.2 M Na_2HPO_4. The relative amounts of synapsin I in the extracts obtained from control or TMT-treated subjects was assayed by back phosphorylation (Forn and Greengard, 1978) in the presence of [γ-^{32}P]ATP and added protein kinase. The standard assay contained 20 μl of neutralized extract, 50 mM piperazine-N,N′bis(2-ethanesulfonic acid), pH 7.4, 10 mM $MgCl_2$, 0.4 mM ethylene glycol bis(β-aminoethyl ether)-N,N′-

tetraacetic acid, 1.0 mM dithiothreitol, 100 nM catalytic subunit and 5 μM ATP (1×10^7 dpm/nmol) in a final volume of 200 μl. The phosphorylation reaction was conducted at 30°C for 30 min and then terminated with an SDS-PAGE sample buffer (O'Callaghan *et al.*, 1983). All samples were heated to 90–95°C before SDS-PAGE.

SDS-PAGE, autoradiography and microdensitometry. The endogenously phosphorylated synaptic proteins and the exogenously phosphorylated acid extracts were subjected to SDS-PAGE as described previously (O'Callaghan *et al.*, 1983). The acrylamide concentration was 6 and 10% in the stacking and resolving gels, respectively. After electrophoresis, the gels were stained for protein with Coomassie brilliant blue R-250 and destained by diffusion in a solution of 30% methanol and 10% acetic acid. Gels were dried between sheets of dialysis membrane using heat (80°C) and reduced pressure. Autoradiographs of the phosphorylated protein bands were obtained using Kodak RP X-ray film in combination with intensifying screens (Dupont Cronex Lightening-Plus). Molecular weight standards (U.S. Biochemical Corporation, Cleveland, OH) were used for estimations of the relative molecular mass of resolved proteins. Autoradiographs of endogenously phosphorylated proteins were scanned with a microdensitometer (LKB Ultrascan Laser Densitometer, LKB Instruments, Gaithersburg, MD). Proteins below M_r = 30 K were not scanned. The background darkness of the autoradiographs was taken as the base line for densitometric determinations. Microdensitometry values obtained were linear with respect to the full range of autoradiographic exposures. Comparisons between control and experimental groups were only made between samples that had been subjected to electrophoresis on the same gel. Autoradiographs of the extracted exogenously phosphorylated proteins were used to localize the M_r = 80 to 85 K synapsin I doublet. These bands were then cut from the dried gels and the absolute amount of ^{32}P incorporation was determined in duplicates by liquid scintillation spectrometry. The amount of phosphorylated synapsin I in extracts of control subjects was linear with respect to the total amount of protein subjected to extraction (data not shown).

Radioimmunoassay of synapsin I and protein III. Three weeks after the administration of saline or TMT (9.0 mg/kg), rats were killed, the hippocampus and frontal cortex (bilateral) were dissected and each tissue sample was immediately homogenized in 10 volumes of 1% SDS heated to 90°C. Synapsin I and a similar neuron-specific phosphoprotein, protein III, were measured by detergent-based radioimmunoassay (Goelz *et al.*, 1981). All samples were analyzed in quadruplicate and assays were performed in 96-well microtiter plates (Becton Dickinson Labware, Oxnard, CA) instead of microfuge tubes as described previously (Goelz *et al.*, 1981). Dilutions of a single control sample were used as a standard for quantification of synapsin I and protein III in samples obtained from saline- and TMT-treated subjects. By comparing the amount of [^{125}I]synapsin I and [^{125}I]protein III immunoprecipitated in the tissue samples of interest to that immunoprecipitated from the sample used as a standard, the specific activity of synapsin I and protein III in a sample relative to that in the standard was obtained. This expression of immunoreactivity (in arbitrary units) is hereafter referred to as relative specific activity. The relative specific activity of synapsin I or protein III for a given sample was multiplied by a factor in order to set the mean relative specific activity of each control group equal to 1.0. The data are presented on both a total and concentration basis, *i.e.*, relative specific activity per hippocampus or frontal cortex and relative specific activity per milligram of hippocampus or frontal cortex protein, respectively. Separate standard curves were generated for each microtiter plate. The length of time for incubation step iii (see Goelz *et al.*, 1981) was 4 hr for synapsin I assays and 12 hr for protein III assays.

Protein assays. Protein was determined according to the method of Bradford (1976) with the exception of the radioimmunoassays, where the method of Lowry *et al.* (1951) was employed. Bovine gamma globulin was used as a standard in the former, whereas bovine serum albumin was used in the latter.

Statistical analyses. Programs on the Statistical Analysis System (SAS, 1982) were used to analyze the data. Data sets in which multiple measures were collected from the same animal were analyzed by multivariate analysis of variance procedures (Morrison, 1967). When treatment effects were found for the multivariate tests individual variables were analyzed by analysis of variance or Student's *t* test for group observations if the comparisons involved only two groups. Significant analyses of variance were followed by appropriate group comparisons using Duncan's multiple range test. In some instances relationships in the data were explored by trend analysis (Winer, 1971). Details of the statistical evaluation for a particular measure appear in the figure or table legend.

Results

Indices of body and brain weight. Acute administration of TMT to the adult rat resulted in a dose-dependent decrease in a number of brain weight parameters without affecting body weight (table 1). Three weeks after administration of TMT (9.0 mg/kg), hippocampal wet weight and total protein were reduced significantly. Although whole brain weights were reduced, this effect cannot be attributed solely to the decrease in hippocampal wet weight because the values for brain weights minus hippocampus were also depressed significantly. None of these effects were transient; 98 days after exposure to TMT (9.0 mg/kg), the wet weights of hippocampus, whole brain and whole brain minus hippocampus were reduced by 30, 8 and 6%, respectively (data not shown). A direct relationship between body weight at dosing and TMT-induced lethality has been reported (Dyer *et al.*, 1982). In agreement with these findings, we observed that the administration of 9.0 mg/kg of TMT did not result in lethality if body weights at dosing were not greater than approximately 225 g.

Histology. The effects of TMT (9.0 mg/kg at 3 weeks) on gross morphology of the hippocampus and frontal cortex are presented in figure 1. In general, our results are consistent with the effects of TMT administered per os (Dyer *et al.*, 1982). The

TABLE 1

Effects of TMT on body and brain weight indices

Rats were killed three weeks after treatment; each value represents the mean ± S.E.M.

N	Dose of TMT	Body Wt. at Dosing	Body Wt. at Death	Δ in Body Wt.	Brain Wts.	Brain Wts. Minus Hippocampus	Hippocampus Wts.	Hippocampus Protein
	mg/kg			g				mg
5	0.0	214 ± 3	328 ± 6	114 ± 6*	1.93 ± 0.03*	1.81 ± 0.03*	0.122 ± 0.004*	5.52 ± 0.25*
5	3.0	222 ± 6	351 ± 11	128 ± 5	1.96 ± 0.04	1.84 ± 0.03	0.123 ± 0.006	5.40 ± 0.17
5	6.0	186 ± 18	305 ± 12	119 ± 14	1.88 ± 0.03	1.77 ± 0.03	0.113 ± 0.003	5.38 ± 0.26
5	9.0	193 ± 11	306 ± 9	112 ± 11	1.78 ± 0.05*	1.69 ± 0.03*	0.092 ± 0.002*	3.96 ± 0.03*

* $F(3,15)$ and P values were 1.35, $P > 1.000$; 5.19, $P < .0117$; 4.28, $P < 0226$; 15.76, $P < .0001$; 9.32, $P < .001$ for body wt., brain wts., brain wts. minus hippocampus, hippocampus wts. and hippocampus protein values, respectively.

* Significantly different from 0.0, 3.0 and 6.0 mg/kg of TMT-exposed groups, $P < .05$.

gross appearance of the hippocampus was characterized by a loss of pyramidal cell neurons, most notably in CA3-CA4 regions, with sparing of the dentate granule cells. Pyramidal cell loss, manifested in diminished density and length of the cell line, was observed in each of the 30 sections prepared from the dorsal hippocampus of each of four TMT-treated rats. Similar changes were not observed in sections obtained from an equal number of control subjects. Two other morphological characteristics distinguished hippocampal sections from TMT-treated subjects: 1) the overall size of the hippocampal formation was reduced, a finding consistent with the effects of TMT on wet weight of this structure and 2) the stratum radiatum and stratum oriens layers were populated with cells of unknown origin, suggesting the possibility of glial proliferation (*e.g.*, see Nadler *et al.*, 1978). In contrast to these observations, the gross morphology of frontal cortex was qualitatively similar for TMT- and saline-treated animals (fig. 1).

Endogenous phosphorylation of synaptic proteins. In initial experiments, the staining pattern and phosphorylation profile of specific synaptic membrane proteins were employed as indices of TMT-induced neurotoxicity. Representative staining patterns and corresponding autoradiographs of synaptic proteins from hippocampus and frontal cortex are shown in figure 2. The pattern of protein staining in samples of hippocampus and frontal cortex did not differ between control and TMT-treated groups. Autoradiographs obtained from the dried gels revealed the substrates of protein kinases present in synaptic membrane fractions. As reported previously (Ueda *et al.*, 1977), the addition of cAMP to the standard phosphorylation assay stimulated the phosphorylation of two bands (M_r = 80 and 85 K) that correspond to the neuron-specific phosphoprotein doublet, synapsin I. In membranes prepared from hippocampus both the basal and cAMP-dependent phosphorylation of synapsin I were reduced as a consequence of the administration of TMT (9.0 mg/kg, 3 weeks). Treatment with TMT also caused a decrease in the endogenous phosphorylation of other synaptic proteins from hippocampus, both in the presence and absence of cAMP. A small but consistent reduction in cAMP-dependent and independent phosphorylation of proteins from frontal cortex was also observed 3 weeks after the administration of TMT. The effects of TMT on endogenous phosphorylation of synaptic membrane proteins were not the result of a direct inhibition of endogenous kinases because addition of TMT (1.0–100 μM) to the assay did not affect phosphorylation (data not shown). Representative time course data for the effects of TMT (9.0 mg/kg) on endogenous phosphorylation are shown in figure 3. These data are presented as microdensitometric scans of autoradiographs obtained from individual subjects. The maximal decrease in phosphorylation of all hippocampal proteins occurred 3 weeks after the administration of TMT. At this time, microdensitometry values for peaks corresponding to synapsin I were reduced in samples obtained from TMT-treated subjects to 35% of the values obtained from saline-treated subjects (data not shown). Endogenous phosphorylation was still depressed 98 days after treatment, suggesting that the effects of TMT on hippocampal phosphopro-

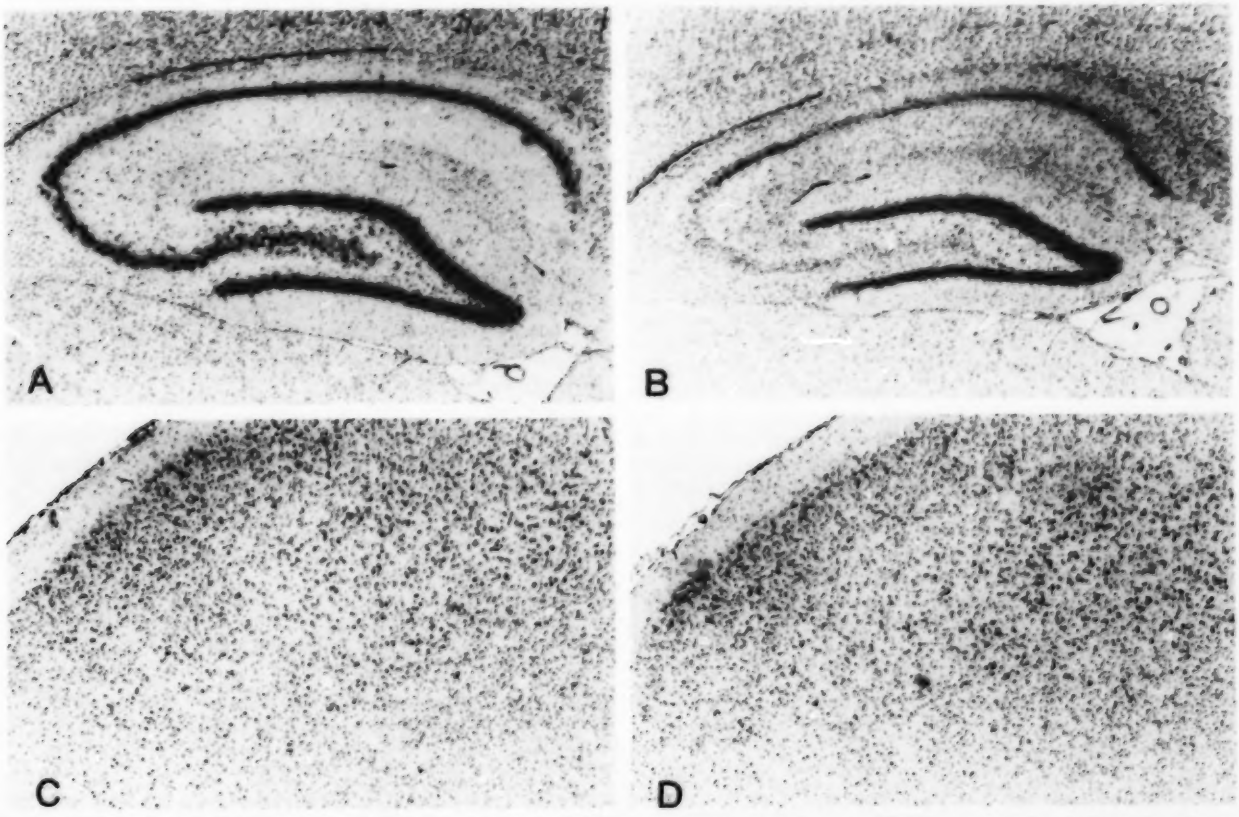

Fig. 1. Morphology of dorsal hippocampus (A,B) and frontal cortex (C,D) three weeks after the acute administration of saline (A,C) or TMT (9.0 mg/kg). (B,D). The magnification is the same for all photomicrographs.

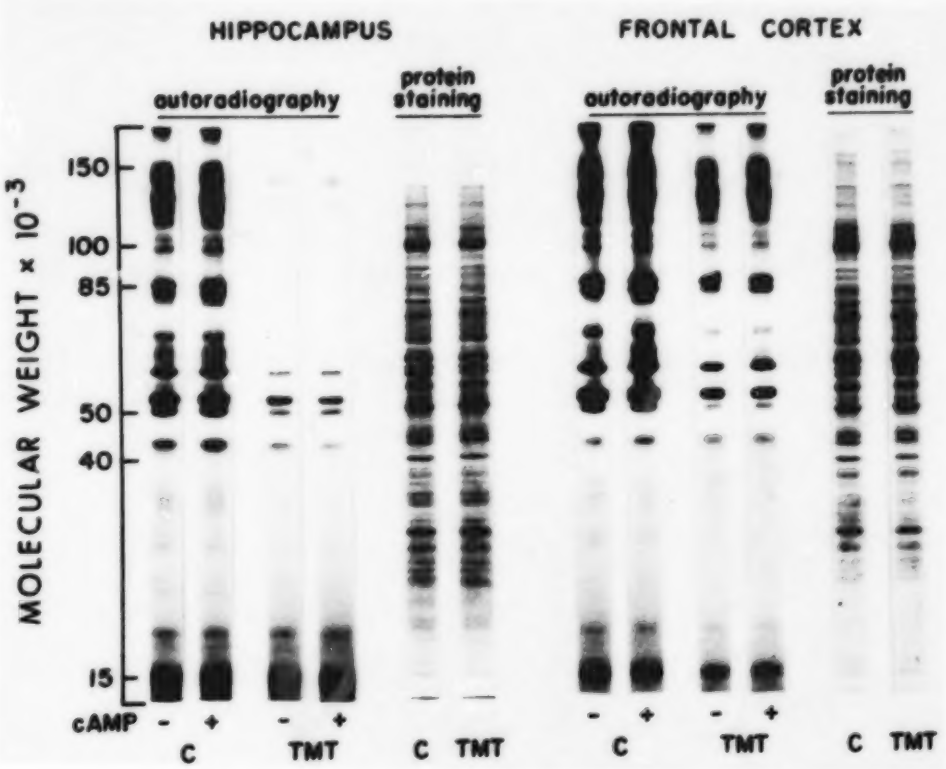

Fig. 2. Effects of TMT on staining and endogenous phosphorylation of synaptic membrane proteins. Crude synaptic membrane fractions were prepared from rats treated with 0.9% saline (C) or (TMT) (9.0 mg/kg) 3 weeks earlier. Proteins were phosphorylated in the absence (–) or presence (+) of 10 μM cAMP.

teins were permanent. The effects of TMT on synaptic membrane phosphoproteins in the hippocampus could not, in general, be extended to the corresponding phosphoproteins in the frontal cortex.

Extraction and phosphorylation of synapsin I. The persistent reduction in phosphorylation of hippocampal synaptic membrane proteins, especially synapsin I, was suggestive of a TMT-induced loss of neuron-specific phosphoproteins. To pursue this lead we employed the back-phosphorylation method to quantify synapsin I in control and TMT-treated subjects. Representative autoradiographs of extracts prepared from hippocampus and frontal cortex are shown in figure 4. Dose-response curves for the effects of TMT on synapsin I in hippocampus and frontal cortex are presented in figure 5. Back-phosphorylation of extracts of hippocampus and frontal cortex showed that TMT caused a decrease in synapsin I in hippocampus but not in frontal cortex (fig. 4). Other hippocampal phosphoproteins, including those with electrophoretic mobilities corresponding to the neuron-specific phosphoproteins collectively referred to as protein III (M_r = 74 and 55 K) (Browning *et al.*, 1982), also appeared to be affected by TMT (fig. 4). The effects of TMT on synapsin I concentration were dose-related and resulted in a loss of as much as 50% of this protein in hippocampus without significantly altering its concentration in frontal cortex (fig. 5). Microdissection of slices of dorsal hippocampus from saline-treated controls did not reveal differences in the concentration of synapsin I among the three regions examined (table 2). TMT caused a significant and nearly comparable decrease in the concentration of synapsin I in all three areas of the hippocampus (table 2).

Radioimmunoassay of synapsin I and protein III. In addition to the back-phosphorylation technique, a detergent-based radioimmunoassay was used to quantify the effects of TMT on synapsin I (table 3). Because the data obtained from the phosphorylated extracts (fig. 4) indicated that protein III as well as synapsin I might be affected by TMT, protein III was also measured by radioimmunoassay (table 3). Administration of TMT (9.0 mg/kg at 3 weeks) resulted in a 61 and 59% decrease in total synapsin I and protein III, respectively, in the hippocampus. This reduction in total synapsin I and protein III did not simply parallel the loss in hippocampal wet weight and total protein that occurred at the high dose of TMT (see table 1) because the values for the concentration of these phosphoproteins in the hippocampus were also reduced significantly (48 and 45%, for synapsin I and protein III, respectively); findings suggestive of a loss of neurons in the hippocampal formation. Radioimmunoassay values for concentration of hippocampal synapsin I were in agreement with those obtained by back-phosphorylation (see table 2; fig. 5). Radioimmunoassay values (total and concentration) for synapsin I and protein III in frontal cortex were not affected as a consequence of the administration of TMT.

Discussion

The neurotoxic effects of acute administration of TMT to the adult rat are characterized morphologically by a loss of pyramidal cells in the hippocampal formation (Brown *et al.*, 1979; Bouldin *et al.*, 1981; Dyer *et al.*, 1982; fig. 1, this paper). Our results demonstrate that these neuropathological effects of TMT are accompanied by dose- and time-dependent decreases in neuronal phosphoproteins, including the synapse-specific

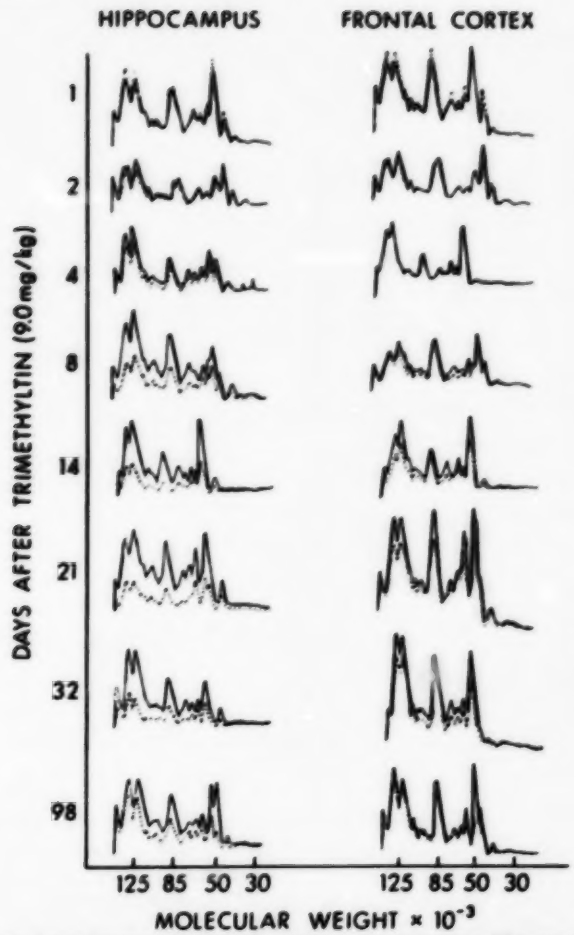

Fig. 3. Time course of the effects of TMT on endogenous phosphorylation of synaptic proteins. The pattern of synaptic membrane phosphorylation from control (0.9% saline) (——) and TMT (9.0 mg/kg)-treated (– – –) rats is represented by densitometric scans of the autoradiographs. All scans were prepared from samples phosphorylated in the presence of 10 μM cAMP. For each time-point and brain region, scans were prepared from each of at least four control and four TMT-treated subjects. A representative scan was then chosen from each group. Relative differences between scans obtained from saline and TMT-treated subjects are valid across days or brain regions. Comparisons of scans across days and brain regions are not valid with respect to absolute values (peak heights or areas).

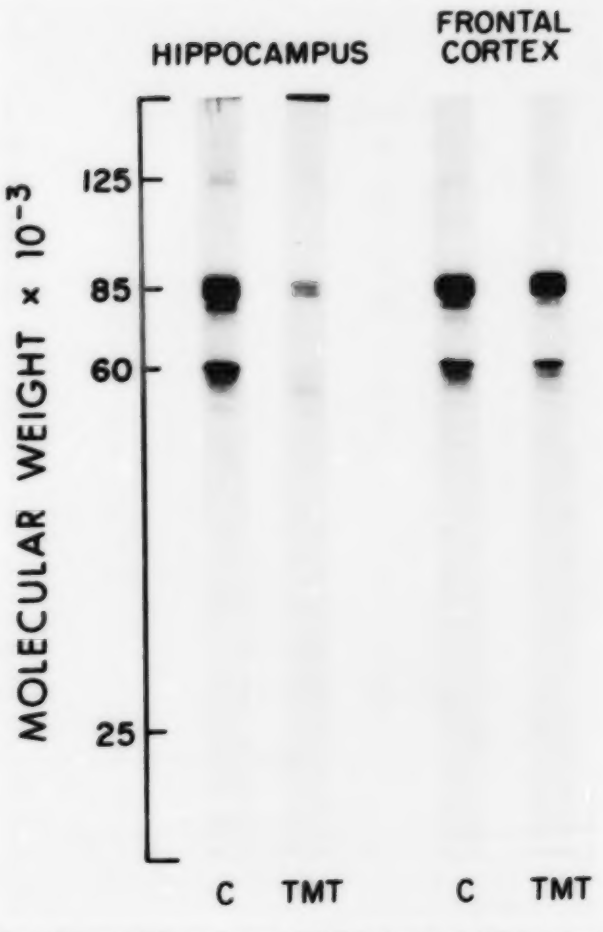

Fig. 4. Effects of TMT on the phosphorylation of extracts of hippocampus and frontal cortex. After preincubation to convert endogenous phosphoproteins to the dephospho form, total particulate fractions were extracted and the proteins in each extract were hosphorylated by the addition of protein kinase and [γ-^{32}P]ATP. Rats were killed three weeks after the administration of 0.9% saline (C) or TMT (9.0 mg/kg).

TABLE 2

Effects of TMT on synapsin I in regions of dorsal hippocampus

Assays of synapsin I were based on phosphorylation of extracts of total membrane proteins. Incorporation of ^{32}P into synapsin I was determined by scintillation spectrometry of slices cut from the dried gels. Rats were killed 3 weeks after the administration of saline (0.9%) or TMT (9.0 mg/kg).

	^{32}P Incorporation into Synapsin I		
	CA1	CA3	Fascia dentata
	pmol/mg protein		
Control	19.6 ± 2.0*	21.9 ± 2.1	18.1 ± 0.9
TMT	8.8 ± 1.3†	9.8 ± 1.7†	10.1 ± 1.4†

* Each value represents the mean ± S.E.M. for five independent observations.
† Significantly different from control, $p < .004$.

phosphoproteins, synapsin I and protein III. Phosphoproteins in the frontal cortex were largely unaffected, findings that are also consistent with the cytopathologic effects of TMT. Thus, by employing a known neurotoxicant to effect a chemically-induced denervation, we have shown that neuron-specific phosphoproteins can serve as biochemical indicators of damage to the neuronal component of the mature CNS. Whereas it is tempting to speculate that the decrease in synaptic phosphoproteins reflects a loss in synapses accompanying neuronal damage, a decrease in either the number of vesicles per synapse, or in the amount of protein per vesicle could also account for our findings (see Goelz *et al.*, 1981).

Where toxicant or lesion-induced cell loss is examined in brain regions composed of few cell types arranged in a uniform pattern (*e.g.*, hippocampus, cerebellum and cerebral cortex), we reasoned that microdissection of tissue slices combined with assays of nervous system-specific proteins would aid in defining the biochemical basis of neurotoxicity. Therefore, we subdivided slices of hippocampus into areas roughly approximating the major internal structures to determine the intrahippocampal distribution of synapsin I in both control and treated animals. Our results indicated that synapsin I concentration in slices prepared from saline-treated subjects did not vary significantly with respect to region. These results are compatible

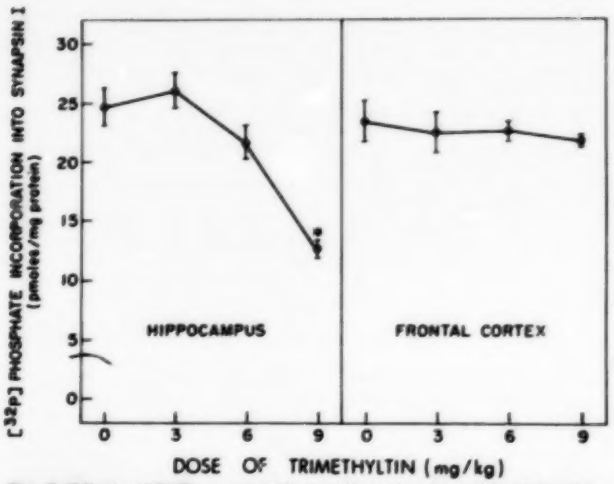

Fig. 5. Effects of TMT on synapsin I in hippocampus and frontal cortex. Assays of synapsin I were based on phosphorylation of extracts of total membrane proteins obtained three weeks after treatment. Each value represents the mean ± S.E.M. for five independent observations. Incorporation of ^{32}P into synapsin I was determined by scintillation spectrometry of slices cut from dried gels. Separate analyses of variance showed an effect of treatment on incorporation of ^{32}P in extract of hippocampus [$F(3,15) = 23.14$, $P < .0001$] but not frontal cortex [$F(3,15) = 0.24$]. In addition, a significant linear trend was found for hippocampus [$F(1,15) = 47.89$, $P < .0001$]. The extract from the hippocampus of the group treated with 9.0 mg/kg of TMT incorporated significantly less ^{32}P than all other groups (asterisk denotes $P < .01$).

with those of immunohistofluorescence studies showing that synapsin I is distributed in a relatively uniform fashion throughout the stratum radiatum and stratum oriens layers of CA1 and CA3 (De Camilli *et al.*, 1983a). Our histological findings indicated that TMT affected pyramidal cells of CA3 to CA4 to the greatest extent; however, cell loss was observed throughout the length of the pyramidal cell line. Because pyramidal cells in regions CA3 to CA4 interconnect all areas of the hippocampal formation (Cotman *et al.*, 1981; Nadler *et al.*, 1980a), their destruction by the neuron-specific toxicant, kainic acid, denervates stratum radiatum and stratum oriens of CA1 and the inner molecular layer of the dentate gyrus (Nadler *et al.*, 1980a, b). Thus, it was not surprising that even more widespread destruction of pyramidal cells by TMT resulted in a reduction in synapsin I concentration that was large in magnitude (50% of control) and that was not confined to a specific region of the hippocampal formation.

Endogenous phosphorylation of synaptic membrane proteins, back-phosphorylation of extracts of total membrane proteins and radioimmunoassays were employed to evaluate the effects of TMT on neuronal phosphoproteins. All three procedures gave similar results; however, only the latter two provided quantitative data with respect to the amount of specific phosphoproteins (synapsin I and protein III). This is due to the fact that changes in endogenous phosphorylation may reflect alterations not only in the amount of specific phosphoproteins, but also in the amount or activity of protein kinases or other factors that affect the net incorporation of phosphate into protein (for a discussion, see Sieghart *et al.*, 1978). Although inconsistencies between the amount and the state of phosphorylation of a given protein were observed in a few instances, it is likely that the effects of TMT on endogenous phosphorylation were largely an expression of changes in the concentration of neuron-specific phosphoproteins. This contention is based on the following lines of evidence: 1) because TMT is known to destroy neurons in limbic structures, its effects on endogenous phosphorylation as well as on values for synapsin I and protein III are consistent with this profile of neurotoxicity; 2) the effects of TMT were not the result of a direct inhibition of the phosphorylation reaction; 3) the time course for the neuropathological effects of TMT coincides with effects on endogenous phosphorylation (Brown *et al.*, 1979; Chang and Dyer, 1983); and 4) the administration of the neuron-specific toxicant, kainic acid, like TMT, results in a decrease in the endogenous phosphorylation of most synaptic membrane proteins (Sieghart *et al.*, 1978). Thus, the TMT-induced decrease in the phosphorylation of several hippocampal proteins in addition to synapsin I (see fig. 2) can be taken as an indication that at least some of these proteins are neuron-specific. Indeed, several neuron-specific phosphoproteins in addition to synapsin I and protein III have been described (Nestler and Greengard, 1983, 1984) and identification of those affected by TMT would seem warranted. If any of these additional proteins prove to be affected by other neuron-specific toxicants, they may be suitable candidates as biochemical indicators of neurotoxicity (for proposed criteria, see O'Callaghan and Miller, 1983).

In agreement with the findings of previous investigators (Bouldin *et al.*, 1981; Chang and Dyer, 1983), our histological examination of hippocampal samples from TMT-treated subjects revealed the presence of cells suggestive of an astrocytic response to neuronal damage. Toxicant- or trauma-induced injury to the CNS often elicits a response characterized by astrocyte proliferation at the site of injury (Nadler *et al.*, 1978; Finger and Stein, 1982; Billingsley and Mandel, 1982). Glial fibrillary acidic protein, a CNS protein specific to fibrous astrocytes (Eng and DeArmond, 1983), has been shown to reflect astrocyte proliferation in response to injury (Latov *et al.*, 1979) and may therefore be an excellent marker for assessing toxicant-induced damage to the CNS (see O'Callaghan and

TABLE 3

Radioimmunoassay of synapsin I and protein III after acute administration of TMT to the rat

Rats were killed three weeks after the administration of saline (0.9%) or TMT (9.0 mg/kg). $N = 5$ for determinations of synapsin I in frontal cortex; $N = 7$ for all other conditions.

	Hippocampus				Frontal Cortex			
	Total		Conc.		Total		Conc.	
	Synapsin I	Protein III	Synapsin I	Protein III	Synapsin I	Protein III	Synapsin I	Protein III
Control	1.00 ± 0.06*	1.00 ± 0.05	1.00 ± 0.05	1.00 ± 0.03	1.00 ± 0.09	1.00 ± 0.04	1.00 ± 0.10	1.00 ± 0.03
TMT	0.39 ± 0.03***	0.41 ± 0.04***	0.52 ± 0.03***	0.55 ± 0.04***	0.98 ± 0.05	0.99 ± 0.10	1.02 ± 0.05	1.03 ± 0.09

* Each value represents the mean relative specific activity ± S.E.M. expressed on a total (per structure) or concentration (per milligram of protein) basis; see "Materials and Methods" for additional details.

*** Significantly different from control, $P < .0001$.

Miller, 1983). Indeed, we have found recently that glial fibrillary acidic protein increases dramatically after the administration of TMT to adult rats (Brock and O'Callaghan, 1984). Because the neuropathological sequelae of exposure to TMT may encompass several CNS cell types, alterations in additional nervous system-specific proteins, both neuronal and glial in origin, may result from the administration of this toxic organometal. We are currently exploring this possibility. Whether the effects of TMT on nervous system-specific proteins can be generalized to broad categories of neurotoxicants must await the outcome of future investigations.

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